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Purification and characterization of yeast myristoyl CoA:protein *N*-myristoyltransferase

(protein fatty acid acylation/protein processing/protein targeting/fatty acyl transferase)

DWIGHT A. TOWLER*, STEVEN P. ADAMS†, SHAD R. EUBANKS†, DEREK S. TOWERY†, EMILY JACKSON-MACHELSKI*, LUIS GLASER‡, AND JEFFREY I. GORDON*§

Departments of *Biological Chemistry and §Medicine, Washington University School of Medicine, St. Louis, MO 63110; †Central Research Laboratory, Monsanto Company, St. Louis, MO 63198; and ‡Department of Biology and Biochemistry, University of Miami, Miami, FL 33156

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ABSTRACT **Myristoyl CoA:protein *N*-myristoyltransferase (NMT) catalyzes the addition of myristic acid to the amino-terminal glycine residues of a number of eukaryotic proteins. Recently, we developed a cell-free system for analyzing NMT activity and have begun to characterize the substrate specificity of this enzyme by using a series of synthetic peptides. We have now purified NMT from *Saccharomyces cerevisiae* to apparent homogeneity. The native enzyme is a 55-kDa protein, exhibits no requirement for divalent cation, and appears to contain a histidine residue critical for enzyme activity. A total of 42 synthetic peptides have been used to define structure/activity relationships in NMT substrates. An amino-terminal glycine is required for acylation; substitution with glycine analogues produces peptides that are inactive as substrates or inhibitors of NMT. A broad spectrum of amino acids is permitted at positions 3 and 4, while strict amino acid requirements are exhibited at position 5. Replacement of Ala^5 in the peptide Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg with Asp ablates the peptide's myristoyl-accepting activity. A serine at this position results in a decrease by a factor of ≈500 in the apparent K_m in the context of three different sequences. Penta- and hexa-peptides are substrates, but with decreased affinity. These studies establish that structural information important for NMT–ligand interaction exists beyond the first two amino acids in peptide substrates and that the side chains of residue 5 play a critical role in the binding of substrates to this enzyme.**

A select subset of cellular proteins are covalently modified with myristic acid either cotranslationally or very soon after completion of polypeptide chain biosynthesis (1–7). Myristate is linked to the amino-terminal glycine residues of these proteins via an amide bond (4, 8–12). The functions of protein N-myristoylation remain to be fully clarified. Myristoylation of the protein tyrosine kinase p60$^{v\text{-}src}$ is required for its localization to cellular membranes and expression of its cell-transforming potential (13, 14). The myristoylated mammalian retroviral gag polyproteins (10, 15) may exploit this cellular targeting mechanism to localize viral budding; Rein *et al.* (16) have demonstrated that myristoylation of the Moloney murine leukemia virus Pr65^{gag} polyprotein precursor was essential for its membrane localization and virus particle formation.

Recently, we determined that both yeast and a murine muscle cell line (BC_3H1) contain an enzyme activity that attaches myristate to the amino-terminal glycine residues of synthetic peptides whose sequences were derived from those of known *N*-myristoyl proteins (5, 17). The protein *N*-myristoyltransferase (NMT) shows a strict specificity for myristoyl CoA as the acyl donor. These initial studies indicated that the substrate specificity of NMT is phylogenetically conserved between lower and higher eukaryotes (17). In this paper, we describe the purification to apparent homogeneity and further characterization of yeast NMT by a variety of standard enzyme inhibitors as well as 27 additional synthetic peptides.

MATERIALS AND METHODS

Cells, Peptides, and Chemicals. The protease-deficient *Saccharomyces cerevisiae* strain JR153 (MATα, trp1, prb1, prc1, pep4-3; see ref. 18) was grown and harvested as described (17). Peptides were synthesized and purified using techniques detailed in an earlier paper (17). Analytic HPLC using a Vydac C-18 column (Vydac Separations Group, Hesperia, CA) indicated that each peptide product used in these studies was >95% pure. Peptide identity was verified by amino acid analysis and by automated sequential Edman degradation with an Applied Biosystems (Foster City, CA) vapor-phase sequencer. The peptide Gly-Ala-Gln-Leu-Ser-Thr-Leu-Gly was a kind gift from G. Grant (Washington University, St. Louis, MO). [^{3}H]Myristic acid (22.4 Ci/mmol; 1 Ci = 37 GBq) and [1-^{14}C]myristoyl CoA (51.2 mCi/mmol) were purchased from New England Nuclear. HPLC grade solvents were obtained from Burdick and Jackson (Muskegon, MI). All other reagents were purchased from Sigma.

Protein determinations and $NaDodSO_4$/PAGE were performed as described (5).

Purification of Yeast NMT. NMT was partially purified (570-fold) as described (17). Three milliliters of the 570-fold purified NMT (0.75 mg/ml) in buffer A [50 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes), pH 6.5/1 mM dithiothreitol] was loaded onto a 1-ml Pharmacia FPLC Mono S column preequilibrated in buffer A. After loading at a flow rate of 1 ml/min, the column was washed for an additional 7 min with buffer A, at which time a 2% per min gradient of buffer B (50 mM Mes, pH 6.5/1 mM dithiothreitol/1 M NaCl) was initiated. NMT activity eluted approximately equally in fractions 19 and 20 (1-min fractions were collected throughout). Fraction 20 was concentrated to 45 μl in a Centricon-30 microconcentrator (Amicon), diluted with 3 ml of buffer A, then rechromatographed on the FPLC Mono S column as described above, but using a shallower gradient (0.5% per min, initiated once 10% buffer B had been reached). Typically, 70–80% of the input NMT activity was recovered from the Mono S column. The enzyme was stored at 4°C in the eluant. NMT activity was slowly lost when stored this way over a period of 3–4 weeks.

NMT Assays. *In vitro* assays of NMT activity were generally performed as described, using reverse-phase HPLC to

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Abbreviations: NMT, myristoyl coenzyme A:protein *N*-myristoyltransferase; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

identify the radiolabeled myristoyl peptide products (5, 17). The peptide substrate specificity of NMT was examined by methods described in detail elsewhere (17). With few exceptions, all myristoyl peptides eluted between 22 and 26 min using our standard HPLC protocol (5). Myristoyl peptides with multiple basic residues (e.g., Gly-Asn-Arg-Ala-Ala-Ala-Arg-Arg) eluted somewhat earlier, between 17 and 20 min. Myristoyl-Gly-Ala-Gln-Leu-Ser-Thr-Leu-Gly eluted at 32 min.

To examine the pH dependence of NMT activity, we used pure NMT (≈1 μg/ml, ≈0.1 μg per assay tube) with 8 μM [1-^{14}C]myristoyl CoA and a variety of buffer systems: 20 mM Mes at pH 6.0, 20 mM sodium phosphate at pH 6.5, 20 mM Hepes at pH 7.0, 20 mM Hepes at pH 7.5, 20 mM Tris·HCl at pH 8.0, 20 mM Tris·HCl at pH 8.5, and 10 mM Tris·HCl/10 mM glycine at pH 9.0. These studies were carried out in the presence of 0.1 M NaCl but in the absence of Mg^{2+}.

For enzyme inhibitor studies, pure NMT (≈0.5 μg of protein in 50 μl of 20 mM potassium phosphate, pH 6.8) was treated for 30 min at room temperature with the reagents and concentrations indicated in Table 1. The treated fractions were diluted 15:110 into NMT assays using 2 μM [1-^{14}C]myristoyl CoA as the acyl donor. Diethylpyrocarbonate-treated samples were adjusted to a final concentration of 20 mM histidine (pH 7) prior to assaying for NMT.

RESULTS

Purification of Yeast NMT. We have previously described a series of four steps that results in a 570-fold purification of yeast NMT (17). A summary of the purification to this stage can be found in the legend to Fig. 1. To estimate the native molecular mass of the enzyme, we performed HPLC gel filtration of this partially purified fraction and assayed for NMT activity. The apparent size of native NMT was determined to be ≈52 kDa (data not shown).

To further purify yeast NMT, the 570-fold enriched preparation was subjected to chromatography on a Pharmacia FPLC Mono S cation exchange column. As shown in Fig. 1*B*, NMT activity was divided approximately equally between fractions 19 and 20. A prominent protein of 55 kDa was also equally distributed between fractions 19 and 20 (Fig. 1*B*). Fraction 20 was rerun on the Mono S column using a shallower gradient. A single Coomassie-stained protein of 55 kDa was recovered, possessing NMT activity that was 11,000-fold enriched from the crude lysate (Fig. 1*C*, lane 6). Fifteen cycles of automated Edman degradation of 0.5 nmol of this protein preparation using a gas-phase sequencer did not yield any detectable phenylthiohydantoin-derivatized amino acids (data not shown), suggesting that NMT may have a blocked amino terminus. When considered together, these observations are consistent with the notion that the 55-kDa protein was both homogenous and represented NMT.

Purified NMT exhibits a slightly alkaline pH optimum (Table 1). The enzyme is inactive at pH 6.0.

A series of standard enzyme inhibitors was examined for their effects on the activity of purified NMT. The enzyme has no apparent requirement for divalent cation; the chelators EDTA and 1,10-phenanthroline had only modest effects on enzyme activity (Table 1). The reagents *p*-hydroxymer-

Table 1. Effects of enzyme inhibitors and pH on yeast NMT activity

Treatment	NMT activity
Control	100
Ethanol (10%)	97
Phenylmethylsulfonyl fluoride (2 mM)	100
Iodoacetamide (10 mM)	95
p-Hydroxymercuribenzoate (1 mM)*	60
Diethylpyrocarbonate (5 mM)*	
20 mM histidine after treatment	1
20 mM histidine during treatment	89
Without Mg^{2+}	95
EDTA (1 mM)†	110
1,10-phenanthroline (0.1 mM)*†	70
pH 6.0	2
pH 6.5	19
pH 7.0	47
pH 7.5	100
pH 8.0	104
pH 8.5	40
pH 9.0	51

The 11,000-fold purified enzyme was treated for 30 min at room temperature with the reagents indicated and then assayed for NMT activity. These data are expressed as percentage of untreated control activity. NMT pH dependence data have been normalized to the rate observed at pH 7.5.
*Stock solutions of these reagents were in ethanol.
†These assays were carried out in the absence of Mg^{2+}.

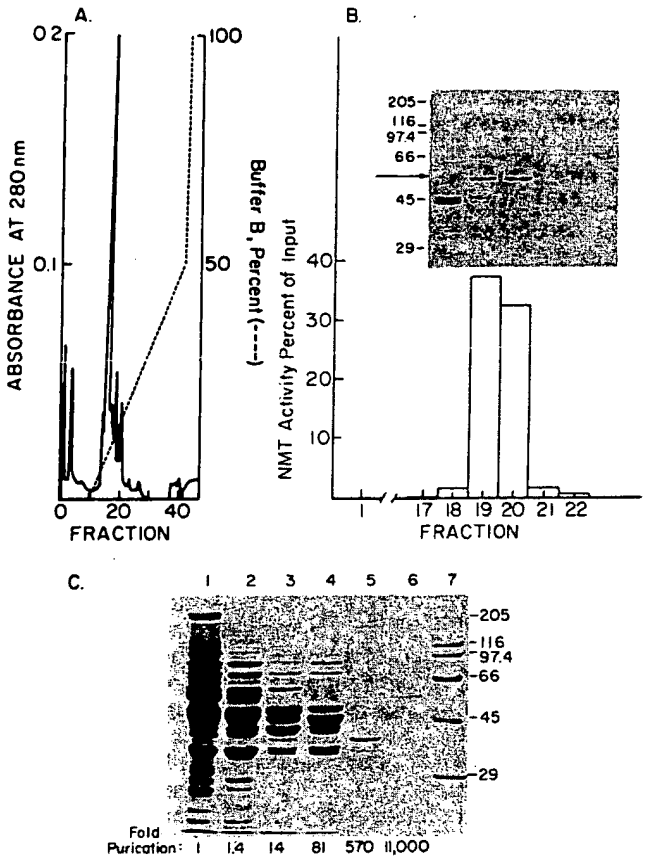

FIG. 1. Purification of yeast NMT. Mono S cation exchange chromatography was performed on the 570-fold purified enzyme fraction as described. (*A*) Protein elution profile as detected by monitoring eluate absorbance at 280 nm. (*B*) NMT activity elution profile. (*Inset*) NaDodSO$_4$/PAGE analysis of proteins present in fractions 18–22. Note the 55-kDa protein (arrow in *B*), which evenly distributes between fractions 19 and 20. (*C*) Protein present in sequentially purified fractions of yeast NMT as analyzed by NaDodSO$_4$/PAGE and Coomassie brilliant blue staining. Lanes: 1, crude lysate; 2, protein precipitated between 51% and 70% saturated ammonium sulfate; 3, 35 mM NaCl eluate from a DEAE-Sepharose CL-6B column; 4, 0.2 M KCl eluate from an agarose-hexane-CoA affinity column; 5, 0.2 M potassium phosphate eluate from a Bio-Rad HTP hydroxylapatite column; 6, 14% buffer B eluate obtained from FPLC Mono S chromatography of fraction 20 (*B*) using a 0.5% per min gradient of buffer B. The purified enzyme has an apparent mass of 55 kDa, and catalyzes the formation of 140 nmol of myristoyl peptide per min per mg of protein when using the parental peptide as a substrate.

curibenzoate, iodoacetamide, and phenylmethylsulfonyl fluoride had only slight effects on NMT, suggesting that the enzyme does not possess modifiable cysteine or serine residues critical for activity. The reagent diethylpyrocarbonate totally inactivated NMT (Table 1). The presence of 20 mM histidine during diethylpyrocarbonate treatment preserved 89% of the enzyme activity but had no protective capacity when added at the end of the treatment period (Table 1). NMT substrates and products were not successful in protecting the enzyme from inactivation by diethylpyrocarbonate (data not shown). Given the relative selectivity of diethylpyrocarbonate for carbethoxylation of histidine residues (19), these data suggest that NMT contains a histidine residue critical for enzymatic activity.

Strategy for Determining the Structure/Activity Relationships for NMT Ligands. Recently, we began examining the amino-terminal substrate specificity of NMT by using 15 synthetic peptides systematically altered from the sequences of known *N*-myristoyl proteins (17). These peptides were examined as substrates and inhibitors of NMT *in vitro*. Using this same methodology, we have extended these initial results by the synthesis of 27 additional peptides designed to probe different aspects of NMT specificity. Analogues of glycine have been substituted for the amino-terminal glycine to determine the structural features of this amino acid important in the binding of peptides to NMT. Other sequence modifications probe structural information present in NMT substrates beyond their amino-terminal residues.¶

Highly Purified NMT Does Not Possess an Intrinsic Methionine Aminopeptidase Activity. The DNA sequences of the known myristoyl proteins $p60^{src}$ (20), murine leukemia virus $p15^{gag}$ (21), and cAMP-dependent protein kinase (22) indicate that the myristoylated glycine is immediately preceded by the initiator methionine residue. Thus, this methionine must be removed prior to myristoylation. To examine the possibility that NMT may also possess this methionine aminopeptidase activity, the peptide Met-Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg was tested as a substrate using pure NMT. This peptide was not an NMT substrate (Table 2). However, the 80-fold purified enzyme preparation could use this peptide as a precursor to [^{3}H]myristoyl-Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg, indicating that an aminopeptidase activity present in this fraction had at least partially copurified with NMT. A peptide possessing acetyl methionine at its amino terminus also failed to function as a substrate for NMT.

The Primary Amino Group of the NH_2-Terminal Glycine Residue Influences Substrate Binding to NMT. Previously, we had documented the amino-terminal requirement for glycine in NMT substrates (17). Neither D- nor L-alanine amino-terminal substitutions produced a functional substrate (17). To further probe this specificity, Gly^1 was replaced with sarcosine (*N*-methylglycine) and propanoate (the isosteric carbo analogue of glycine). In the absence of the primary amine, neither of these peptides was functional as a substrate (Table 2). Moreover, neither of these peptides was bound by NMT with any significant affinity, as indicated by their inability to inhibit myristoylation of the parental substrate (Table 2). Thus, the amino-terminal group of glycine is involved in the binding of substrates to the enzyme. The positioning of this primary amine is also critical, since a peptide with a β-alanine substitution at the amino terminus was not bound by NMT (Table 2). A peptide with a Pro^2 substitution is not an NMT ligand (Table 2). This observation suggests a requirement for correct presentation of the amino group of Gly^1.

Table 2. Substrate specificity of NMT

	K_m, mM	V_{max}*, %	K_i†, mM
Substitutions at position 1			
Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg‡	0.06	100	
Met-§		—	
AcMet-		—	
Me-Gly-		—	±
Prp-			±
β-Ala			±
Substitutions at position 2			
-CHA-		—	+
-Pro-		—	±
-Phe-‡		—	+
Substitutions at position 3			
-Arg-	0.43	75	
-Phe-	0.06	121	
-Pro-	0.5	8	
Substitutions at position 4			
Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg‡	0.06	100	
-Pro-	0.3	60	
Substitutions at position 5			
-Ser-	0.0001	3	0.00002
-Asp-		—	—
-Phe-	0.085	4	
-Pro-		—	—
-Ser-‡	1.7	50	
-Ser- -Ser-	0.003	34	
-Tyr-‡		—	0.16
-Tyr- -Ser-	0.3	3	+
Gly-Ala-Gln-Ala-Ala-Ala-Arg-Arg	1.4	56	
Gly-Ala-Gln-Leu-Ser-Thr-Leu-Gly	0.003	23	
Gly-Ala-Arg-Ala-Ala-Ala-Arg-Arg	0.83	5	
Gly-Ala-Arg-Ala-Ser-Val-Ser-Gly	0.43	121	
Substitutions at position 6			
Gly-Ser-Ser-Lys-Ser-Lys-Pro-Lys‡	0.04	43	
-Pro-Lys-Asp-Pro-Ser‡		—	—
-Pro		—	—
Peptide size requirements			
Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg‡	0.06	100	
Gly-Asn-Ala-Ala-Ala-Ala	1.3	92	
Gly-Asn-Ala-Ala-Ser	0.9	40	
Gly-Tyr-Ala-Ala-Ser		—	
Gly-Ala-Arg-Ala-Ala-Ala-Arg-Arg	0.83	5	
Gly-Ala-Arg-Ala-Ala-Ala-Ala-Arg-Arg	0.3	5	

Peptide sequences are given either in their entirety or only where they differ from the parental sequence Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg. AcMet, acetyl methionine; Me-Gly, sarcosine; Prp, propanoyl; CHA, cyclohexylalanine.

*V_{max} data are expressed as the percentage maximal rate observed for the parental peptide substrate. —, No activity as an NMT substrate could be detected (V_{max}, <1%).

†±, Weak (<25%) inhibition of NMT; +, >25% inhibition under the assay conditions described in ref. 17.

‡Data for these peptides were taken from ref. 17.

§This peptide did not function as a substrate using pure yeast NMT, but it could be used as a precursor to myristoyl-Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg by less-purified fractions.

NMT Can Accommodate a Spectrum of Amino Acids at Positions 3 and 4 in Peptide Substrates. Previous studies had indicated that the amino acids permitted at position 2 were also restricted, with only uncharged residues (preferably with polar side chains) being active as substrates. Peptides with hydrophobic residues were good inhibitors and poor substrates. In contrast to the relatively restricted amino acid requirements at positions 1 and 2, position 3 can accommodate Phe, Gln, Arg, and Pro (Table 2) as well as Glu, Ala, and Ser residues (17). However, the kinetic parameters of these various peptides differ; peptides with uncharged residues at position 3 generally exhibit a lower K_m (Table 2; ref. 17),

¶The apparent K_m values obtained for peptide substrates using 80-fold purified NMT were essentially the same as those obtained with the homogeneous enzyme (data not shown). The 80-fold purified enzyme was used in these studies because of its stability and ease of preparation. This fraction had also been used in our initial investigations of peptide substrate specificity (ref. 17).

while the peptide with Pro^3 exhibited a relatively poor catalytic rate (Table 2).

A great deal of flexibility is also exhibited in the residues permitted at position 4 in NMT substrates; Ala, Lys, Leu, and Pro residues can be accommodated (Table 2).

Residues at Positions 5 and 6 Play a Role in Substrate Recognition by NMT. We have noted that important structural information appears to be present in substrate sequences beyond the ultimate and penultimate residues; deletion of a single amino acid at position 6 (Lys^6) in a substrate peptide corresponding to the $p60^{src}$ amino terminus produced an inactive analogue (5, 17). One potential mechanism whereby the deletion of a residue within a substrate sequence could produce an inactive analogue would be that this change introduces a sterically unacceptable conformation in residues beyond the deletion. To examine this possibility for the $p60^{src}$ peptide variant mentioned above, the hexapeptide Gly-Ser-Ser-Lys-Ser-Pro was tested as a substrate or inhibitor of NMT. The sequence of this peptide corresponds to the first six amino acids of the inactive Lys^6-deleted $p60^{src}$ variant. As shown in Table 2, this peptide was not bound by NMT. The inability of this peptide to be bound by NMT cannot be attributed to its size, since another hexapeptide and even a pentapeptide can function as substrates (see below). Thus, the residue present at position 6 can influence substrate binding to NMT.

Inspection of the amino-terminal sequences of the five known *N*-myristoyl proteins yields no obvious consensus other than the amino-terminal glycine residue. However, three of these proteins—$p60^{src}$ (12), calcineurin B (8), and cytochrome b_5 reductase (11)—contain a serine at residue 5, while the other two proteins—cAMP-dependent protein kinase (9) and murine leukemia virus p15 gag (10)—contain alanine and threonine at this position. The relatively restricted spectrum of amino acids present at position 5 in these myristoyl proteins suggested an important role for residue 5 in substrate recognition by NMT. We investigated this hypothesis by replacing Ala^5 with Ser, Asp, and Phe in the sequence Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg. As shown in Table 2, the Ala^5 to Ser^5 substitution resulted in a remarkable decrease (by a factor of 600) in the K_m for the peptide, indicating that NMT binds this new peptide with much higher affinity. This substitution also resulted in a reduction in V_{max} by a factor of 33. In contrast, the peptide with Asp^5 was totally inactive as a substrate and as an inhibitor of the enzyme. A peptide with phenylalanine at position 5 produced a substrate with affinity comparable to the original sequence. In this context, both the Ser^5- and Phe^5-substituted peptides exhibited catalytic rates ≈3% that of the Ala^5 parent.

To further substantiate the dramatic increase in peptide binding with the Ser^5 substitution, we introduced a serine at position 5 in the sequence Gly-Ser-Ala-Ala-Ala-Ala-Arg-Arg (a substrate peptide with the relatively high K_m of 1700×10^{-6} M). This Ser^5 for Ala^5 substitution again produced a great decrease (by a factor of 570) in the K_m for the peptide (Table 2). Although less straightforward a comparison, a similar effect can be seen by inspecting the K_m for the peptide Gly-Ala-Gln-Ala-Ala-Ala-Arg-Arg (1400×10^{-6} M) compared to Gly-Ala-Gln-Leu-Ser-Thr-Leu-Gly (3×10^{-6} M). When these data are considered together, it is clear that residues at position 5 can play an important role in binding peptide substrates to NMT.

The presence of Ser^5 in NMT substrates does not always yield peptides with a very low K_m. Gly-Asn-Glu-Ala-Ser-Tyr-Pro-Leu (corresponding to the calcineurin B sequence; ref. 8) and Gly-Ala-Arg-Ala-Ser-Val-Ser-Gly (corresponding to the HTLV-III gag sequence; ref. 23) were both good NMT substrates possessing Ser^5 and relatively high K_m values (2300 and 430×10^{-6} M, respectively; ref. 17 and Table 2). These higher K_m values most probably reflect the preference of NMT for substrates with uncharged residues at position 3 (see above). Moreover, the Ser^5 substitution in the purely inhibitory peptide Gly-Tyr-Ala-Ala-Ala-Ala-Arg-Arg (17) did not produce a peptide with high affinity. Nonetheless, this new peptide did function as an NMT substrate, although with a relatively poor catalytic rate (Table 2).

Shorter Peptides Are Bound with Significantly Lower Affinity. To establish the peptide length requirements for substrate recognition by NMT, a hexapeptide (Gly-Asn-Ala-Ala-Ala-Ala) and a pentapeptide (Gly-Asn-Ala-Ala-Ser) were examined as substrates. As shown in Table 2, both of these peptides are recognized by NMT, but with greatly decreased affinities for the enzyme.

An octapeptide substrate elongated to a nonapeptide by insertion of an alanine residue between positions 6 and 7 produced a substrate with slightly better affinity for NMT. When considered together, these data indicate that residues beyond the first six amino acids may influence ligand binding to NMT.

The Myristoyl Peptide Product Can Compete for the Myristoyl CoA Binding Site. The interaction of enzymatic product with NMT was investigated by examining the ability of myristoyl-Gly-Ser-Ser-Lys-Ser-Lys-Pro-Lys to inhibit myristoylation of Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg. The myristoyl peptide product decreased only the apparent V_{max} for the peptide substrate and increased the apparent K_m of myristoyl CoA (Fig. 2), hallmarks of noncompetitive and competitive inhibition, respectively (24). The secondary plots in Fig. 2 indicate that the myristoylated peptide product binds to the myristoyl CoA binding site with a $K_i = 5\text{–}15 \times 10^{-6}$ M. However, since the K_m of myristoyl CoA is 0.1–0.4 $\times 10^{-6}$ M (Fig. 2; ref. 17), the product (myristoylated peptide) is bound with at least 1/10th the affinity of the myristoyl CoA substrate.

DISCUSSION

NMT purified from yeast appears to be a 55-kDa monomer. Given the marked specificity of NMT for acyl donor and acyl acceptor (Table 2; ref. 17), the mechanism whereby an enzyme of relatively simple subunit composition obtains such selectivity promises to be interesting.

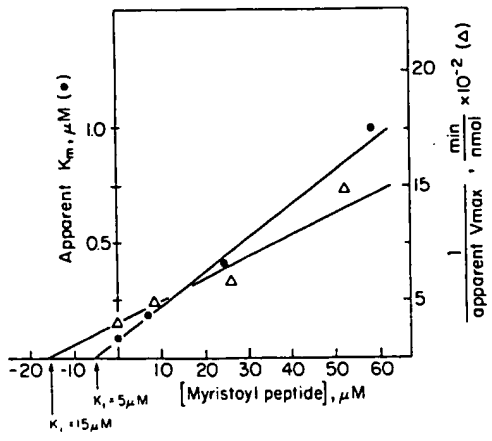

FIG. 2. Inhibition of NMT by the myristoyl peptide product. The effects of increasing myristoyl peptide (product) concentrations on the apparent K_m and V_{max} values for peptide substrate and myristoyl CoA were examined by the standard assay protocol. Analysis of these data by double-reciprocal plots indicated that the product inhibited noncompetitively with respect to peptide substrate but inhibited competitively with respect to myristoyl CoA (data not shown). Shown are secondary plots for noncompetitive inhibition with the peptide substrate (Δ) and competitive inhibition with myristoyl CoA (●). The myristoylated peptide product binds to NMT with a K_i of ≈10×10^{-6} M.

The data presented here suggest that a histidine residue is critical for activity. The dramatic increase in NMT activity from pH 6.5 to pH 7.5 (Table 1) may be due to the titration of a critical histidine, since histidine residues are known to possess approximately neutral pK values.

The presence of a blocked amino terminus and a slightly alkaline pH optimum is consistent with cytoplasmic orientation of NMT. Enzyme activity is present in both crude membrane and soluble fractions prepared from yeast (5) as well as BC_3H1 murine muscle cells (unpublished observations). Thus, NMT may be a peripheral membrane protein or associated with polysomes.

The absence of a methionine aminopeptidase activity intrinsic to purified NMT indicates that *in vivo* another enzyme must first remove the initiator methionine from nascent apomyristoyl proteins before NMT can recognize them as substrates. In cells, these two activities might coexist as part of an amino terminus processing complex, thus ensuring stoichiometric acylation of myristoyl proteins.

NMT exhibits a high degree of selectivity for the sequence of its peptide substrates. The *in vitro* data gathered to date (Table 2; ref. 17) can be summarized as follows. (*i*) NMT has an absolute requirement for amino-terminal glycine in its peptide substrates. This amino acid's small side chain and primary amino group are critical in ligand binding to the enzyme. (*ii*) A limited spectrum of residues are allowed at position 2 in peptide substrates. Neutral amino acids (Asn, Ser, Gln, Ala, Val) are allowed at this position; negatively charged residues are not. Peptides with aromatic or bulky hydrophobic residues at position 2 are NMT ligands but are very poor substrates. Pro^2 is not allowed, presumably because it affects the orientation of Gly^1. (*iii*) A wider spectrum of amino acids can be accommodated at positions 3 and 4 in NMT substrates. Peptides with uncharged residues at position 3 are generally bound with greater affinity. A Pro^3 substitution is tolerated less well than a Pro^4 substitution. (*iv*) The dramatic effects of position 5 substitutions demonstrate the importance of residues in this position in NMT–ligand interactions. Ser^5 is a highly favored residue, while a negatively charged residue (Asp^5) produces a peptide totally inactive as a substrate. The spectrum of residues present at this position in peptide substrates is remarkably similar to that present at position 2. Peptides with Pro^5 or Pro^6 residues are nonfunctional as NMT substrates. (*v*) Residues beyond the first 6 amino acids may play a role in substrate recognition by NMT. The precise structural requirements in this region of NMT ligands remain to be elucidated.

The *in vitro* peptide substrate data collected to date (Table 2; ref. 17) should prove useful in identifying potential *N*-myristoyl proteins specified by eukaryotic cDNA sequences (accounting for the presence of the initiator methionine), since the substrate specificity of NMT is phylogenetically conserved (17). The cDNA for $p56^{LSTRA}$ kinase, a myristoyl protein awaiting the chemical identification of *N*-myristoyl glycine (25), possesses codons for Gly-Cys-Val-Cys-Ser-Ser-Asn-Pro immediately following the initiator methionine codon (26). In light of the NMT substrate specificity, the presence of Gly^1, Cys^2, and Ser^5 in this sequence strongly supports the proposal that $p56^{LSTRA}$ is myristoylated on this amino-terminal glycine (26). Several interesting candidates for N-myristoylation are the HTLV-III p17 gag (23), the guanine nucleotide binding proteins $G_{\alpha i}$ (27, 28) and transducin (29, 30), the hepatitis B virus "Pre-S1" antigen (31), and the *yes*- and *src*-related protooncogene c-*syn* (32). Note that an octapeptide corresponding to the amino terminus of the HTLV-III gag protein (Gly-Ala-Arg-Ala-Ser-Val-Ser-Gly; ref. 23) functions as a substrate for yeast NMT *in vitro* (Table 2). However, the possibility remains that some myristoyl proteins may be acylated at a cryptic site exposed only after a specific proteolytic cleavage and would thus be missed if only the amino-terminal sequences of primary translation products were considered as possible myristoylation sites.

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